**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant: HARPOLD et al. Group Art Unit: 1812

Serial No.: 07/938,154 Examiner: Ulm, J.

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For: HUMAN NEURONAL NICOTINIC ACETYL CHOLINE RECEPTOR COMPOSITIONS AND METHODS EMPLOYING SAME

**DECLARATION PURSUANT TO 37 C.F.R. §1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, STEVEN BRADLEY ELLIS declare as follows:

1) I am a co-inventor of the above-identified U.S. Patent Application Serial No. 07/938,154 as well as the parent application U.S. application Serial No. 07/504,455, which is now U.S. Patent No. 5,369,028.

2) I was employed by THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC. (hereinafter SIBIA) from December 1981 through July, 1995. I served as a Molecular Cellular Biologist from 1981 through 1985, as a Research Scientist from 1985 through April 1992, as a Senior Research Scientist from 1992 to April 1994, and as Senior Research Fellow from 1994 to 1995.

3) As the details presented below indicate, it was not a straightforward endeavor to obtain DNA encoding any of the human neuronal nicotinic acetylcholine receptors (NAChRs) despite the fact that DNA encoding certain rat nicotinic acetylcholine receptor subunits were known. In attempting to clone the subunits, a number of difficulties were encountered, including: a) the difficulty in obtaining suitable human neuronal tissue samples from which to prepare libraries; b) the differences between the distribution of receptor subtypes in humans and rats; and c) the unpredictability of such tissue samples with respect to the types of receptors that are expressed.

4) The following description of cloning of each of the receptor subunits evidences the above-described difficulties and the differences between the rat NAChR subunits and human NAChR subunits.

## ISOLATION OF DNA ENCODING A HUMAN NAChR $\alpha_2$ SUBUNIT

RNA encoding the rat $\alpha_4$ subunit is abundant in rat thalamus. Consequently, in order to isolate DNA encoding a human NAChR $\alpha_4$ subunit, a human thalamus library was screened with DNA encoding the rat $\alpha_4$ subunit as a probe. Four positive DNA clones $\alpha_{2.1}$, $\alpha_{2.2}$, $\alpha_{2.11}$, and $\alpha_{2.13}$ were obtained. The four clones, however, did not have substantial homology with rat $\alpha_4$-encoding but were more homologous to rat $\alpha_2$-encoding DNA. Clones $\alpha_{2.1}$ and $\alpha_{2.13}$ were spliced to produce a full length $\alpha_2$-encoding clone. The 3′ end of human clone $\alpha_{2.1}$ is about 81% homologous to rat $\alpha_2$-encoding DNA and only 43% homologous to the DNA clones encoding the rat $\alpha_3$, $\alpha_4$ and $\beta_2$ subunits. The human clone $\alpha_{2.11}$ is only about 75% homologous to the DNA encoding the rat $\alpha_2$ subunit. Thus, the resulting clone is less than about 80% homologous to the DNA encoding rat $\alpha_2$.

## ISOLATION OF DNA ENCODING A HUMAN NAChR $\alpha_3$ SUBUNIT

To isolate the NAChR $\alpha_3$ subunit the following five human cDNA libraries were screened using the rat $\alpha_3$ clone as a probe:

| Library screened with rat $\alpha_3$ probe | Positives |
|---|---|
| SIBIA prefrontal cortex library ($9.4 \times 10^5$ recombinants) | 0 |
| Clontech Temporal Cortex library ($9.2 \times 10^5$ recombinants) | 0 |
| ATCC Basal Ganglia library ($5 \times 10^5$ recombinants) | 0 |
| ATCC Spinal Cord library ($5 \times 10^5$ recombinants) | 0 |
| ATCC Brain Stem library ($6.6 \times 10^5$ recombinants) | 7 |

Thus, the only clones obtained, clones, $\alpha_{3.1}$ -$\alpha_{3.7}$, were obtained from the brain stem library. In rats, however, $\alpha_3$ is not abundant in the brain stem. The rat $\alpha_3$ encoding DNA had been isolated from a rat PC12 cell-derived cDNA library and the rat $\alpha_3$ subunit appears to be a CNS-associated subunit that is abundantly expressed in the thalamus. Since RNA encoding the $\alpha_3$ subunit is abundant in rat thalamus, a human thalamus library was screened with $\alpha_{3.6}$. Only four positive clones $\alpha_{3.11}$- $\alpha_{3.14}$ were obtained.

The full-length human nicotinic acetylcholine receptor $\alpha_3$ subunit was ultimately isolated by screening a cDNA library prepared from mRNA isolated from dibutyryl cyclic AMP-induced IMR32 (human peripheral nervous system neuroblastoma) cells, using rat-human hybrid $\alpha_3$ probe (550 nucleotides containing the DNA encoding the rat signal sequence and the first five codons of the mature protein and the remainder was from the human $\alpha_3$ clone $\alpha_{3.5}$). Although IMR32 cells reportedly express few, if any, functional nicotinic acetylcholine receptors (see, e.g., Gotti et al. ((1986) Biochem. Biophys. Res. Commun. 137: 1141-1147, and Clementi et al. (1986) J. Neurochem. 47: 291-297), twenty-four positive clones were obtained. Among the twenty-four clones, two clones had no homology to any known NAChR subunit-encoding DNA and one clone $\alpha_{3.24}$ encodes a full-length $\alpha_3$ subunit.

The human $\alpha_{3.5}$ clone was used to probe northern blots of RNA from dibutyryl cAMP-induced IMR32 cells and from uninduced IMR32 cells. A 3.2 kb transcript, which is of sufficient size to encode the human $\alpha_3$ mRNA, was detected in both RNA samples. An additional 4.2 kb transcript was detected in the RNA from the induced cells but not from the uninduced cells.

Human $\alpha_3$-encoding DNA and a pool of 5' oligomers were subsequently used to screen another library prepared from dibutyryl cAMP induced IMR32 cells. Sequence analysis of the positive clones revealed that three of them were partial clones identical to corresponding portions of the $\alpha_3$ clone, but that a fourth positive clone KE$\alpha_{3.6}$ was different. The 5' end of the clone KE$\alpha_{3.6}$ has only 40% sequence identity with the other $\alpha_3$ clones and the 3' end has 100% sequence identity with the other clones. In addition, the 5' end of KE$\alpha_{3.6}$ does not contain a splice acceptor site, suggesting that it does not represent an incompletely spliced transcript. It appears that KE$\alpha_{3.6}$ is the product of alternative splicing of the $\alpha_3$ gene.

**ISOLATION OF DNA ENCODING A HUMAN NAChR $\beta_2$ SUBUNIT**

Although $\beta_2$ is not abundant in rat prefrontal cortex, screening a prefrontal human brain cortex library ($9.4 \times 10^5$ recombinants; prepared in house at SIBIA) using rat $\beta_2$ cDNA yielded a positive clone, $\beta_{2.1}$, which contains about 80% of the coding portion of the human gene. $\beta_{2.1}$ also was used to screen a temporal cortex library ($9.2 \times 10^5$ recombinants; Clontech). No positives were found in this cortex library, despite the success in obtaining $\beta_{2.1}$ from the first cortex library using the rat clone. Since mRNA encoding $\beta_2$ is abundant in rat thalamus, a human thalamus library ($1 \times 10^6$ recombinants, prepared at SIBIA) was screened with the $\beta_{2.1}$ clone. Only one positive clone, $\beta_{2.2}$ was obtained. A human thalamus library ($1.8 \times 10^6$) was then constructed using oligo dT and human $\beta_2$ specific primers to prepare the cDNA and screened using the 5' end of the rat $\beta_2$ clone as a probe. Three clones $\beta_{2.5}$ -$\beta_{2.7}$ that hybridized to the probe were identified. A full-length $\beta_2$ clone was constructed by splicing partial clones $\beta_{2.1}$, $\beta_{2.2}$ and $\beta_{2.7}$.

An IMR32 library ($1 \times 10^6$ recombinants) was screened with the human $\beta_2$ probe. As discussed above, human $\alpha_3$ appears to be abundantly expressed in IMR32 cells lines, and publications discussing the rat receptors indicate that a functional channel requires an $\alpha$ and $\beta$. Since $\alpha$ was expressed in IMR32 cells, it was expected that $\beta$ would also be expressed. Only four hybridizing clones were identified, however, and have not been completely characterized. Northern analysis of IMR32 RNA, using rat $\beta_2$ DNA as probe, did not detect any hybridizing mRNA, thus rendering the precise nature of the $\beta$ clones unclear. It appears that $\beta_2$ may be expressed at very low levels in IMR32 cells.

**ATTEMPTS TO ISOLATE DNA ENCODING A HUMAN NAChR $\alpha_4$ SUBUNIT**

After failing to isolate human $\alpha_4$ clones from a thalamus library, a human brain stem library was screened using low stringency wash conditions with the rat $\alpha_4$ DNA probe. The eighteen most intensely hybridizing positive clones were analyzed. None of eighteen clones encoded an $\alpha_4$ subunit.

Since in vitro hybridization studies of RNA isolated from rat brain tissue demonstrate that the $\alpha_4$ transcript is highly expressed in the habenula (see, e.g., Goldman et al. (1987) Cell 48: 965), a human habenula library was screened under high stringency wash conditions using the rat $\alpha_4$-encoding DNA. The only hybridizing DNA obtained was a human $\alpha_2$ clone.

**ATTEMPTS TO ISOLATE DNA ENCODING A HUMAN NAChR $\alpha_5$ SUBUNIT**

A randomly primed library prepared from RNA isolated from dibutyryl cAMP-induced IMR32 cells was screened under conditions of high stringency with a 1.1 kb fragment including the 5' end of the coding portion of rat $\alpha_5$-encoding DNA. No hybridizing clones were obtained. High stringency conditions were used in order to avoid isolating $\alpha_3$ clones.

It has been reported (Chini et al. (1992) Proc. Nat'l. Acad. Sci. 89: 1572, which is attached hereto) that human $\alpha_5$-encoding clones have been obtained from a library produced from RNA isolated induced IMR32 cells using a roughly 500 bp rat $\alpha_5$ probe that encompasses the 3' coding region of the rat clone and screening under low stringency conditions.

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Steven Bradley Ellis
STEVEN BRADLEY ELLIS

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6362-9380